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GLTSCR2 contributes to the death resistance and invasiveness of hypoxia-selected cancer cells

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ABSTRACT

Tumor hypoxia may be an indicator of poor survival in cancer patients. Thus, an understanding of the molecular mechanism responsible for hypoxic tumor selection is essential to gain further insight into tumor biology. Our aim in this study was to investigate whether hypoxia-responsive GLTSCR2 contributes to death resistance and increased invasiveness of hypoxia-selected glioblastoma cells. We found that repeated hypoxia downregulates p53-upstream regulator, GLTSCR2, which resulted in increased death resistance and invasive potential of glioblastoma cells. Restoration of GLTSCR2 expression suppressed the malignant potential of hypoxia-selected cells. Our results indicate that GLTSCR2 participates in hypoxia-induced malignant potential.

1. Introduction

GLTSCR2/Pict-1 is a nucleolar protein involved in critical cellular processes such as cell cycle regulation, DNA damage response, and apoptosis [1–3]. Nucleolar GLTSCR2, in response to ribosomal stresses or DNA damages, is redistributed diffusely throughout the nucleoplasm, where it participates in cell cycle regulation by upregulating p53 through inhibition of MDM2-mediated ubiquitination of p53, or controls DNA damage response via the phospho-H2AX-dependent pathway [3,4]. In addition, several reports have identified GLTSCR2 as a tumor suppressor that is downregulated and mutated in brain tumor cells; furthermore, knockdown of its expression promotes anchorage-independent growth of cells, a characteristic feature of malignant cells [5,6]. Recently, however, it has been reported that GLTSCR2 acts as an oncogene and that increased expression of GLTSCR2 transcript is associated with poor survival in colon and esophageal cancers [7]. These discrepant results imply that this protein has multiplex activities associated with cancers and that complicated signaling networks of proteins are involved in GLTSCR2-mediated tumor suppressive or oncogenic processes. The effects of GLTSCR2 on the progression of malignant tumors and underlying molecular mechanisms have yet to be determined.

An adequate supply of oxygen from the vascular network is essential for rapid tumor growth. However, tumor cells are often exposed to a hypoxic environment due to uncoordinated excessive growth of tumor parenchyma relative to vascular connective tissue, which limits further growth of the tumor mass [8]. In contrast, hypoxia may act as a poor survival factor because highly aggressive tumor cells that can overcome the unfavorable oxygen condition can proliferate exclusively in the tumor mass [9]. Therefore, an understanding of the phenotypic changes induced by chronic hypoxia and the underlying molecular mechanisms is essential to develop appropriate and effective cancer treatment modalities and to gain further insight into tumor biology.

Previously, we reported that GLTSCR2 sensitizes cells to hypoxic injury [10], suggesting a putative role for GLTSCR2 in chronic hypoxic selection of tumor cells. Our aim in this study was to investigate whether GLTSCR2 contributes to the death resistance and invasiveness of hypoxia-selected glioblastoma cells. Using hypoxia-resistant T98G cells selected by repeated exposure to hypoxia and reoxygenation, we show that downregulation of GLTSCR2 caused by chronic hypoxia plays a crucial role in aggressive behavior of hypoxia-selected glioblastoma cells.

Abbreviations: ARF, alternative reading frame; GLTSCR2, glioblastoma tumor suppressive candidate region gene 2; JNK, c-jun N-terminal kinase; MDM2, murine double minute 2; NPM, nucleophosmin; Pict-1, Protein interacting with C-terminus-1; PTEN, phosphatase and tensin homolog; RPL11, ribosomal protein L11

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2. Methods

2.1. Cell culture and construction of hypoxia-resistant T98G cells

T98G and LN18 glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's (DMEM) supplemented with 10% fetal bovine serum. Construction of hypoxia-resistant clones was performed by exposure of cells to repeated hypoxia-reoxygenation cycles. Cells in a degassed medium were exposed to 0.5% O_2 balanced with 5% CO_2/94.5% N_2 in a hypoxic chamber (In vivo2, Ruskinn, UK) for 12 h, followed by incubation under normal culture conditions for recovery. Death resistance to hypoxia was determined by the trypan blue exclusion method after each cycle.

2.2. Antibody and reagents

The anti-GLTSCR2 antibody we used was described in a previous study [3]. Anti-p53 and anti-tubulin antibodies, and secondary antibodies conjugated with horseradish peroxidase, were purchased from Abcam (Cambridge, UK). Unless specified otherwise, reagents were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). SP600125, PD98095, and VO-OHpic were purchased from EMD Chemicals Inc. (MA, USA).

2.3. Tet-off adenoviral-mediated system construction

The assembly and production of recombinant adenovirus was performed according to the manufacturer's instructions (Adeno-X™ Tet-Off Expression System 1, Clontech Laboratories, Inc., CA, USA), as described previously [2].

2.4. Knockdown of GLTSCR2

GLTSCR2-knockdown was established using a Lentivector-based small hairpin RNA (shRNA) system according to the manufacturer's instructions (GeneCopoeia Inc., Rockville, MD, USA). The GLTSCR2-targeted sequence was 5′-GAG ACC GGT TCA AGA GCT T-3′, and the scrambled shRNA sequence was 5′-CGA TAC TGA ACG AAT C-3′. For lentiviral-mediated gene transfer, these sequences were cloned into the psiLv-H1 shRNA expression vector and transfected into HEK293 cells for pseudoviral packaging. Supernatants were collected and purified viral particles were titered and used to infect cells.

2.5. Western blot analysis and proximity ligation assay (PLA)

Western blot analysis to detect GLTSCR2 and p53 was performed as described previously [3]. PLAs were carried out using a Duolink® II detection kit (Olink Bioscience, Uppsala, Sweden) as described previously [11]. Briefly, cells in chamber slides were incubated with rabbit anti-GLTSCR2 and mouse anti-p53 antibodies and then secondary antibodies conjugated to unique DNA probes (PLA probe MINUS and PLA probe PLUS) were added. Ligation and circularization of the DNA was followed by a rolling circle amplification step, and reactions were detected by a complementary Cy3-labeled DNA linker. Slides were evaluated using an LSM 510 META confocal microscope (Zeiss, Jena, Germany).

2.6. Invasion assay

Invasion assays were carried out using a cell invasion kit (EMD Milipores, MA, USA) according to the manufacturer's protocol. Briefly, 1×10^4 cells were plated in a Matrigel-coated transwell invasion chamber and incubated at 37 °C for 24 h. Non-invading cells were removed by wiping the upper side of the membrane of the transwell. Invading cells were fixed with methanol and stained with hematoxylin. We performed three independent invasion assays in triplicate. On average, six randomly selected fields were counted under a light microscope.

2.7. Immunofluorescence

For immunostaining, cells were fixed with 4% paraformaldehyde and incubated sequentially with primary antibody overnight at 4 °C and secondary antibody for 2 h at 4 °C. Cells were viewed under a Nomarski differential interference contrast (DIC)-equipped inverted confocal microscope (META 510, Zeiss, Germany) after nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI).

2.8. Statistics

Statistical analyses were performed using SPSS software, version 12.0 (SPSS, Chicago, IL, USA). Data were analyzed with two-tailed, unpaired Student's *t*-tests or ANOVA. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. c-jun N-terminal kinase (JNK)-dependent nucleoplasmic redistribution of GLTSCR2 in hypoxia

Most, if not all, nucleolar proteins shuttle between the nucleolus and nucleoplasm during periods of genotoxic or ribosomal stress through the various signaling pathways [12–14]. Thus, we investigated whether GLTSCR2 translocates to the nucleoplasm in response to hypoxia. T98G glioblastoma cells were exposed to 0.5% hypoxia for 12 h and immunostained using anti-GLTSCR2 antibody to evaluate its intracellular distribution. In most cells, nucleolar GLTSCR2 was released to the nucleoplasm after hypoxic exposure (Figs. 1A and B). The results of the experiments using LN18 glioblastoma cells were similar to those obtained with T98G cells (Fig. S1). We tested which signaling molecules are involved in this delocalization of GLTSCR2 under hypoxic exposure. T98G cells were treated with c-jun N-terminal kinase (JNK) inhibitor SP600125, extracellular signal-regulated kinase (ERK) inhibitor PD98095, or phosphatase and tensin homolog (PTEN) inhibitor VO-OHpic, followed by exposure to 0.5% hypoxia for 12 h, and immunostained using anti-GLTSCR2 antibody. Figs. 1A and suplementary data S2 show that inhibition of JNK by SP600125 or JNK inhibitor III, or transfection with JNK-dominant negative (JNK DN) plasmid suppressed the hypoxia-induced nucleoplasmic mobilization of GLTSCR2 while inhibition of ERK or PTEN failed to inhibit nucleoplasmic translocation of GLTSCR2 in T98G cells. Our results indicate that in T98G glioblastoma cells, GLTSCR2 translocates to the nucleoplasm in response to hypoxia via a JNK-dependent pathway.

3.2. GLTSCR2 expression is downregulated in hypoxia-selected tumor cells

To investigate the effects of chronic hypoxia on total GLTSCR2 expression level, we constructed hypoxia-selected clones of T98G cells (HRT98G) by repeated hypoxia-reoxygenation cycles as described in the methods section. After twelve cycles of hypoxia-reoxygenation, cell death rate and invasiveness of HRT98G cells were determined by the trypan blue exclusion method and invasion assays using Matrigel-coated transwell invasion chambers, respectively. As shown in Figs. 2A, 2B, and S5, we noted significant decrease in hypoxia-induced cell death and increased invasive potential of HRT98G cells compared to parental T98G cells. In

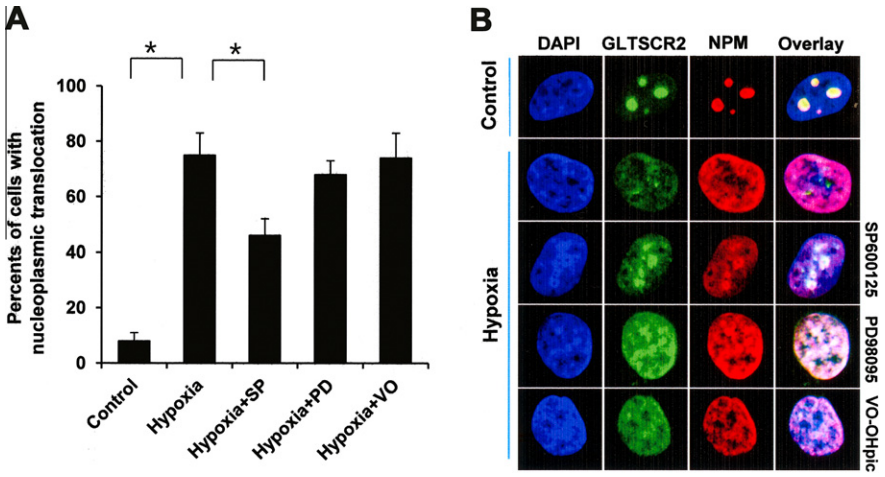

Fig. 1. Hypoxia induces nucleoplasmic translocation of GLTSCR2 in a JNK-dependent manner. (A) T98G cells were exposed to 0.5% hypoxia for 12 h in the presence or absence of JNK inhibitor, SP600125 (20 μM; SP), ERK inhibitor, PD98095 (25 μM; PD), or PTEN inhibitor, VO-OHpic (100 nM; VO), and were then immunostained to detect GLTSCR2 expression. Cells were viewed under a confocal microscope. Data represents means ± SDs of three independent experiments; $^{*}P < 0.05$. (B) Cells described in (A) were stained with rabbit anti-GLTSCR2, mouse anti-nucleophosmin (NPM) antibodies, and DAPI. Representative images in T98G cells are shown.

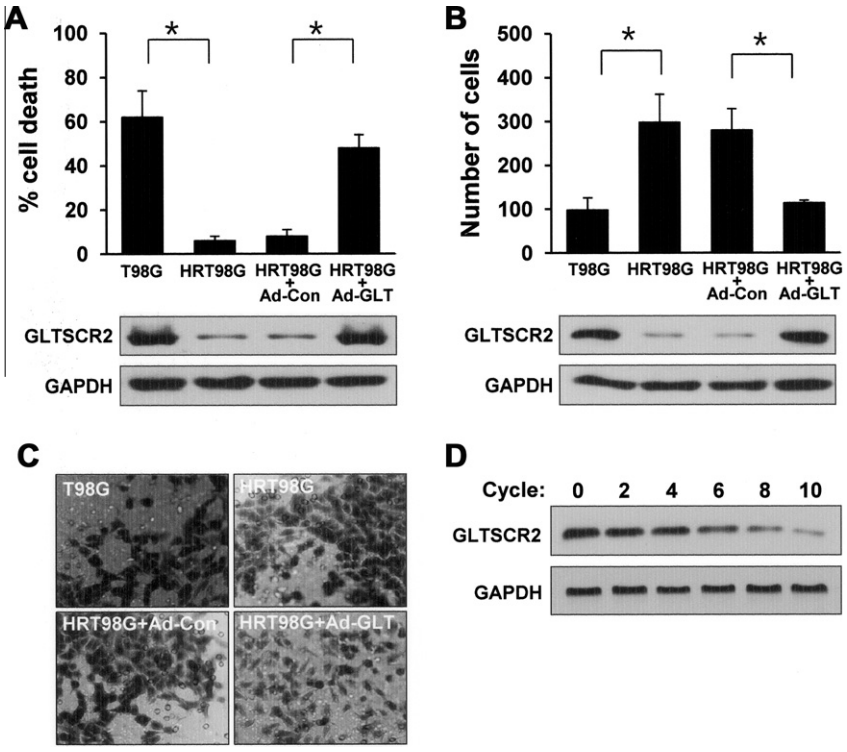

Fig. 2. Restoration of GLTSCR2 expression increases hypoxia sensitivity and reduces the invasive potential of hypoxia-selected glioblastoma cells. (A) Parental (T98G) or hypoxia-selected (HRT98G) cells were infected by GLTSCR2-expressing adenovirus (Ad-GLT) or control virus (Ad-Con) for 24 h and were exposed to 0.5% hypoxia for 12 h. Then, cell death rates were determined by the trypan blue exclusion method. Data are means ± SDs of three independent experiments; $^{*}P < 0.05$. Cell lysates were subjected to Western blot analysis to determine GLTSCR2 expression and apoptosis (lower panel). (B) Parental or HRT98G cells infected by Ad-GLT or Ad-Con for 24 h were plated in a Matrigel-coated transwell invasion chamber and incubated at 37 °C for 24 h. Invading cells were fixed with methanol, stained with hematoxylin, and counted in six randomly selected fields under a light microscope. Data represents means ± SDs of three independent experiments; $^{*}P < 0.05$. Cell lysates were subjected to Western blot analysis to determine GLTSCR2 expression (lower panel). (C) Representative light microscopic images from (B) are shown (X100). (D) Cell lysates after every two cycles of hypoxia-reoxygenation were subjected to Western blot analysis to determine GLTSCR2. GAPDH was used as a loading control.

parallel with the increased viability and invasiveness of HRT98G cells, GLTSCR2 expression gradually decreased as the number of hypoxia-reoxygenation cycles increased (Fig. 2D). Although our data reveal that chronic hypoxia-reoxygenation cycles induced greater malignant potential and GLTSCR2 downregulation in T98G cells, it is not clear that phenotypic changes were caused by suppression of GLTSCR2. Thus, we restored GLTSCR2 expression level in HRT98G cells to the level observed in parental T98G cells by infecting these cells with GLTSCR2-expressing adenovirus (Ad-GLTSCR2). Controlled restoration of GLTSCR2 expression to the level seen in the parental cells increased the cell death rate against hypoxia and decreased invasiveness of HRT98G cells (Fig. 2A and B), albeit not completely, suggesting that downregulation of GLTSCR2 by repeated hypoxia-reoxygenation is one of the factors that contrib-

utes to death-resistance against hypoxia and enhanced invasive potential.

3.3. Downregulation of GLTSCR2 increases the death resistance against hypoxia and invasiveness of glioblastoma cells

Data shown above indicate that chronic hypoxia results in downregulation of the expression of GLTSCR2, which acts as a survival factor against chronic hypoxia. Hence, we determined whether suppression of GLTSCR2 expression changes the phenotypes of parental T98G cells to hypoxia-selected HRT98G cells. T98G cells were infected by lentivirus delivering shRNA targeted to GLTSCR2 (shGLT-T98G cells) or scrambled shRNA (shSCR-T98G cells), and stably infected cells were constructed by puromycin selection (Figs. 3A and B, lower panel). We observed that shGLT-T98G cells were resistant against hypoxia compared to shSCR-T98G cells (Fig. 3A, upper panel). In addition, invasion assay using Matrigel-coated transwell invasion chambers revealed that downregulation of GLTSCR2 potentiated the invasive growth of T98G cells (Figs. 3B, upper panel and C). Interestingly, exogenous expression of GLTSCR2 by Ad-GLTSCR2 restored the sensitivity to hypoxia in shGLT-T98G cells and reduced the invasive potential almost to the level seen in shSCR-T98G cells (Fig. 3A and B). The same experiments were performed with LN18 cells. The results of experiment with the LN18 cells were similar to those of the T98G cells (Fig. S3). Taken together, our data demonstrate that knockdown of GLTSCR2 expression acts as an aggressive factor resulting in death resistance and invasive growth of tumor cells under hypoxia.

3.4. Suppression of GLTSCR2 fails to upregulate p53 under hypoxic condition

Previously, we reported that GLTSCR2 functions as an upstream positive regulator of p53 [4]. P53 is a well-known cell cycle regulator that plays an important role in hypoxia-induced cell cycle arrest [15]. Thus, to investigate the mechanism underlying enhanced cell death resistance by hypoxia-induced GLTSCR2 downregulation, we explored whether hypoxia affects GLTSCR2-p53 axis using a proximity ligation assay (PLA) to detect in situ protein complex formation between p53 and GLTSCR2. Detection is mediated via rolling circle amplification between adjacent, oligonucleotide-coupled secondary antibodies [16]. T98G, HRT98G, and shGLT-T98G cells were exposed to normoxia or 0.5% hypoxia for 12 h and then PLA was performed. As shown in Figs. 4A and B, the number of GLTSCR2-p53 complexes was significantly increased in T98G cells under hypoxic condition while not in HRT98G and shGLT-T98G cells. In line with PLA, we observed the increase of p53 expression level in hypoxia-exposed T98G cells (Fig. 4C), but not in HRT98G and shGLT-T98G cells, suggesting that GLTSCR2 plays a crucial role in hypoxia-induced p53 upregulation. Restoration of p53 expression by ectopically expressed GLTSCR2 in HRT98G and shGLT-T98G cells reinforces our results. Taken together, we propose that GLTSCR2 participates in p53 upregulation in hypoxia-exposed cells.

4. Discussion

Glioblastoma is a rapidly growing and highly aggressive malignant tumor of the brain [17]. The rapid growth of glioblastomas increases the likelihood of tumor hypoxia caused by uncoordinated parenchymal overgrowth relative to the growth of vascular connective tissues. Previously, we reported that GLTSCR2 expression is suppressed to a greater extent in grade IV astrocytic tumors than in grade I–II astrocytic tumors [5], suggesting that GLTSCR2 may be involved in greater malignant phenotype of astrocytic tumors. However, the links among tumor hypoxia in glioblastoma, greater malignant change, and GLTSCR2 expression are not known. Here,

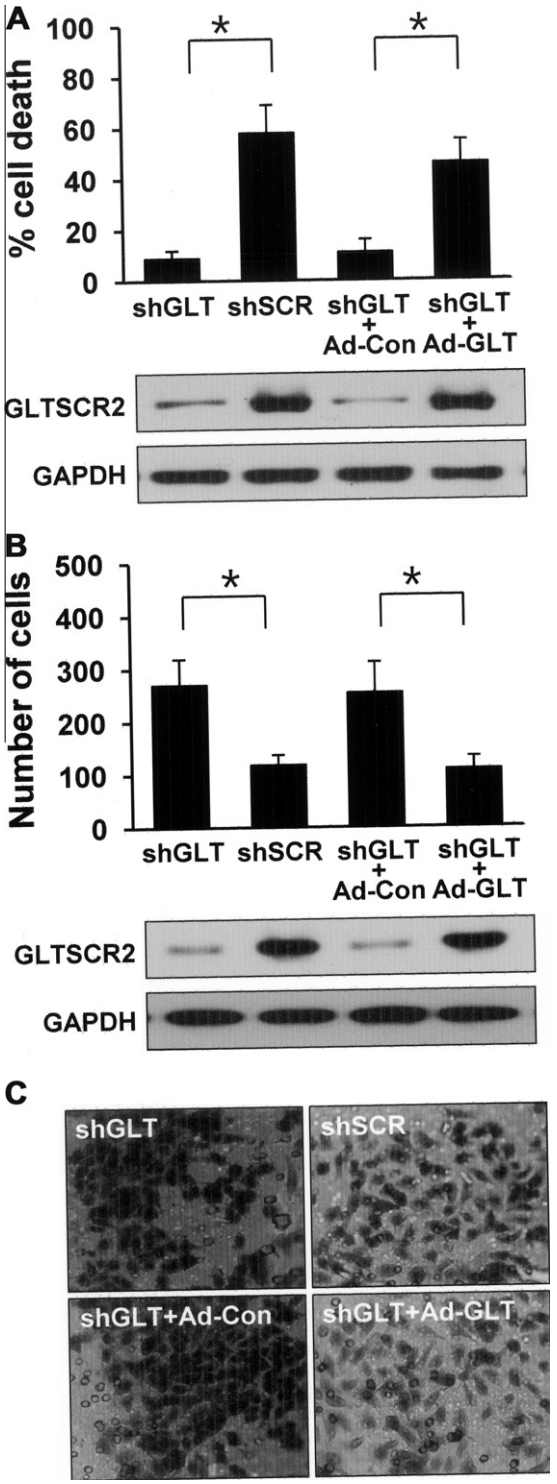

Fig. 3. Downregulation of GLTSCR2 induces death resistance and invasiveness in hypoxia-selected glioblastoma cells. (A) T98G cells were infected by lentivirus containing shRNA targeted to GLTSCR2 (shGLT) or scrambled shRNA (shSCR), and stable cells were constructed by puromycin selection. These stable cells were infected by GLTSCR2-expressing adenovirus (Ad-GLT) or control virus (Ad-Con) for 24 h and were then exposed to 0.5% hypoxia for 12 h. The cell death rate was determined by the trypan blue exclusion method. Data are means ± SDs of three independent experiments; $^{*}P < 0.05$ (upper panel). Cell lysates were subjected to Western blot analysis to determine GLTSCR2 expression (lower panel). (B) Cells from (A) were subjected to a Matrigel invasion assay. Invading cells were fixed with methanol and stained with hematoxylin. On average, six randomly selected fields were counted under a light microscope. Data are means ± SDs of three independent experiments; $^{*}P < 0.05$ (upper panel). Cell lysates were subjected to Western blot analysis to determine GLTSCR2 expression (lower panel). (C) Representative light microscopic images from (B) are shown (×100).

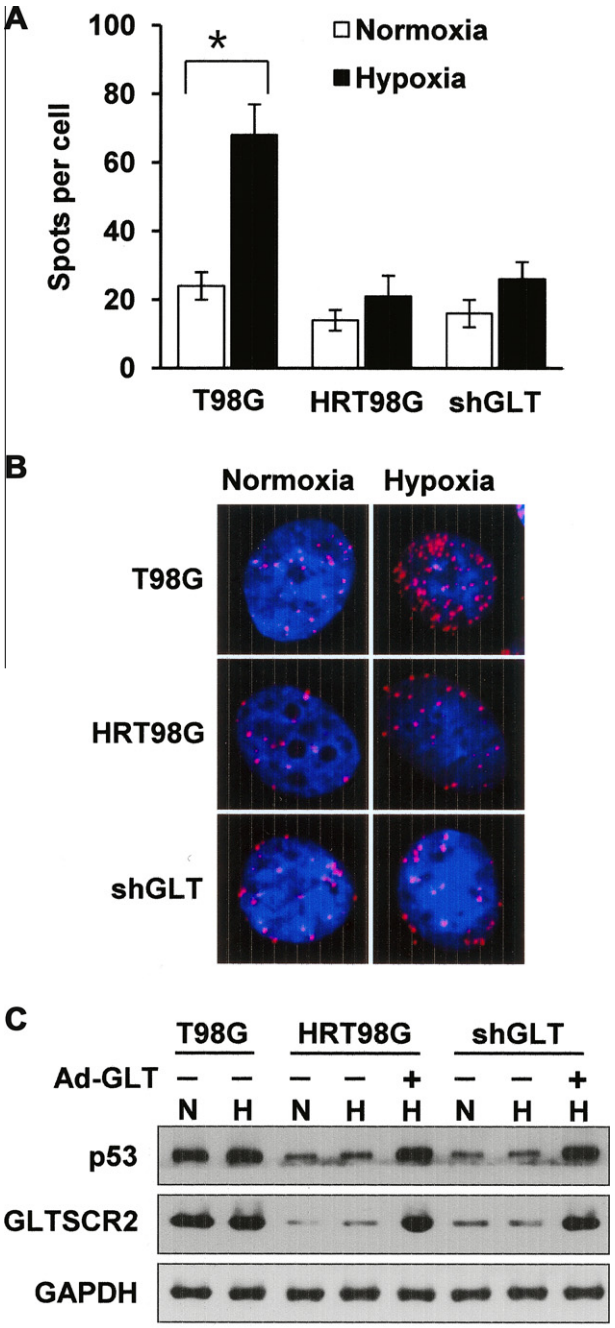

Fig. 4. Hypoxia induces upregulation of p53 and increases GLTSCR2-p53 interactions. (A) Cells were exposed to normoxia or 0.5% hypoxia for 12 h, and a proximate ligation assay using anti-GLTSCR2 and anti-p53 antibodies was performed. The numbers of spots in at least 200 cells were counted; the average numbers of spots per cell from three independent experiments are shown. (B) Representative PLA images from (A) are shown. (C) T98G cells were infected by GLTSCR2-expressing adenovirus (Ad-GLT) or control virus for 24 h, and then exposed to normoxia (N) or 0.5% hypoxia (H) for 12 h. Cell lysates were subjected to Western blot analysis for the detection of GLTSCR2 and p53 and the results were normalized to GAPDH expression.

we investigated the role of GLTSCR2 in the death resistance and invasiveness of hypoxia-selected glioblastoma cells.

Recent findings provided evidence that chronic hypoxia promotes an aggressive cancer cell phenotype [9,18,24,25]. Kato et al. established and characterized a hypoxia-resistant cancer cell line derived from gastric carcinoma by exposure to chronic hypoxia [18]. These hypoxia-resistant gastric cancer cells showed increased migratory and invasive activities and higher metastatic potential than parental cancer cells. However, the molecular mechanism underlying phenotypic changes in cancer cells after exposure to hypoxia remains unknown. Several studies suggested that loss of p53 function plays an important role in cell survival under chronic hypoxic conditions. Inactivating mutations and loss of heterozygosity in p53 is involved in regulating the adaptive response of tumor cells to hypoxia by enhancing their survival and the release of pro-angiogenic factors [19]. The greater malignant clones harboring genetic defects in p53 might be selected for by exposure to hypoxia [20]. Our study addressed the change to a greater malignant phenotype under chronic hypoxia in a glioblastoma cell line, and the associated molecular mechanism. Hypoxia induces translocation of nucleolar GLTSCR2 to the nucleoplasm, where the GLTSCR2-p53 interaction is promoted, thereby activating the anti-proliferative function of p53 [4]. However, hypoxia-resistant cells have a decreased GLTSCR2 level and a remarkably diminished number of GLTSCR2-p53 complexes compared to parental cells (Fig. 4). These findings suggest that inefficient upregulation of p53 might be caused by downregulation of GLTSCR2 in HRT98G and contribute to the rapid, uncontrolled proliferation of hypoxia-exposed glioblastoma cells. Therefore, our data indicate that GLTSCR2 plays a role in the death resistance and invasiveness of hypoxia-selected tumor cells, which might be mediated by the GLTSCR2-p53 interaction.

Our study showed that chronic hypoxia increases the invasive activity of glioblastoma cells and the controlled ectopic expression of GLTSCR2 decreases the enhanced invasive potential. The decreased sensitivity to hypoxia in HRT98G cells compared to parental cells was restored by ectopic GLTSCR2. However, rescue of hypoxia sensitivity and invasiveness by exogenous GLTSCR2 in HRT98G is not sufficient to reach the level seen in the parental cells (Figs. 2A and B). These data suggest that other molecules or mechanisms are involved in hypoxia-induced death resistance and invasiveness. Recently, anti-apoptotic proteins such as Bcl-2, Bcl-X_L, and p-ERK have been shown to participate in hypoxia-induced phenotypic changes [21–23]. In line with the previous result, Bcl-2 and Bcl-XL expression was increased in HRT98G cells (Fig. S4). Therefore, Bcl2 family proteins and ERK pathway may be other candidate molecules involved in hypoxia-induced death resistance and invasiveness.

We showed that the GLTSCR2-p53 interaction was enhanced under hypoxic condition in T87G cells but not in HRT98G and shGLT-T98G cells. Ectopically expressed GLTSCR2 in HRT98G and shGLT-T98G cells rescued p53 expression. These results suggest that GLTSCR2 participates in p53 upregulation in cells exposed to hypoxia. However, a recent study reported that ribosomal protein L11 (RLP11) was released to the nucleoplasm in GLTSCR2-knockout mouse embryonic stem cells, where it inhibited mdm2-mediated degradation of p53, indicating that GLTSCR2 acts as a negative regulator of p53 in this cell type [7]. The discrepant results concerning p53 regulation by GLTSCR2 suggest that divergent pathways are involved in GLTSCR2-p53 regulation. Nevertheless, limited upregulation of p53 due to GLTSCR2 suppression contributes to the aggressive behavior of glioblastoma cells. The molecular mechanism responsible for GLTSCR2 downregulation by chronic hypoxia remains to be determined.

Our results indicate that in T98G cells, GLTSCR2 translocates to the nucleoplasm in response to hypoxia via a JNK-dependent pathway. A recent study demonstrated that JNK activity regulates the distribution of nucleolar proteins. [26] UV, which is the robust activator of JNK, dispersed B23 and ARF from the nucleolus almost completely. However, preincubation with SP600125 before UV irradiation inhibited the translocation of ARF from the nucleolus. This study demonstrated that c-Jun, which is a primary JNK substrate interacts with B23/ARF complexes and changes its subnuclear

localization. It was suggested that JNK activity elevates c-Jun levels, thus increasing the association between c-Jun and B23, and that the phosphorylation of $Thr^{91/93}$ by JNK enhances the translocation of c-Jun and the associated B23 out of the nucleolus. JNK substrates such as c-Jun and JunB can directly control the distribution of GLTSCR2 by interaction with GLTSCR2, or indirectly regulate GLTSCR2 localization by interaction with other nucleolar proteins such as B23 or ARF. The JNK-dependent mechanism of GLTSCR2 translocation still remains to be elucidated.

Collectively, we showed that repeated chronic hypoxia-reoxygenation cycles decreased the expression of GLTSCR2 in glioblastoma cells, which increased their resistance to hypoxic injury and their invasiveness. Because hypoxic selection results in a poor response to treatment, cancer recurrence, and increased metastasis, our results may be helpful in developing appropriate and effective glioblastoma treatment modalities.

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Conflict of interests

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.064.

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